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# Effects of Poor Maternal Nutrition on GH, IGF-I, IGFBP-2 and -3, Insulin, and Leptin Concentrations in Pregnant Ewes

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**Effects of poor maternal nutrition on GH, IGF-I, IGFBP-2 and -3, insulin,  
and leptin concentrations in pregnant ewes**

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## Literature Review

### **Developmental Programming**

The once abstract notion that a transient stressor could instill a permanent phenotypic change in a developing fetus now enjoys a much more credible reputation. Human epidemiological studies have revealed instances in which periods of malnutrition or toxin exposure lead to developmental defects in children of exposed pregnant women, and in some cases in subsequent generations (Barker, 2004). Animal studies using rodent and livestock models have expanded on these findings, reporting permanent morphologic and functional changes in animals whose dams were exposed to various stressors during prenatal development (Allen et al., 2002; Park et al., 2008; Long et al., 2010; Zhang et al., 2011; Giussani et al., 2012). This adoption of permanent structural and physiological changes in response to external stimuli during fetal or early postnatal development is known as developmental programming (Reynolds et al., 2009). Phenotype is determined by extrinsic environmental stimuli in addition to genetic information, and this contradicts the notion that genotype is a rigid blueprint. Rather, developmental plasticity allows for the derivation of more than one phenotype from a single complement of genes (Barker, 2004). Under the theory of developmental plasticity, the object of this process is to mold an individual whose traits are best suited to its specific environment.

Environmental factors include nutrient availability, temperature, the presence of toxins or other compounds, and oxygen availability. The intrauterine environment is the source of environmental stimuli during fetal development and has been shown to affect the phenotype of progeny (Allen et al., 2002; Qasem et al., 2012; Sharma et al., 2012). Embryo-transfer studies have demonstrated that the intrauterine environment of the recipient dam may have a greater

impact on offspring phenotype than genetic contribution of the embryo donor. Sharma et al. (2012) performed such an experiment, using breed as the controlling factor. Single embryos of large-breed Suffolk ewes and small-breed Cheviot ewes were transferred within and between breeds, creating four treatment groups (Suffolk in Suffolk; Suffolk in Cheviot; Cheviot in Cheviot; Cheviot in Suffolk). Large-breed lambs born to small-breed ewes weighed less at birth and had smaller body dimensions compared with their counterparts carried to terms in large-breed ewes. However, this effect was not observed in small-breed lambs transferred to large-breed ewes. Allen et al. (2002) performed a similar experiment but used Thoroughbred horses as the large-breed genotype and Ponies as the small-breed genotype. Similar to the observations made by Sharma et al. (2012), large-breed Thoroughbreds born to small-breed Ponies were lighter at birth compared with those born to Thoroughbred mares. In contrast to Sharma et al. (2012), Allen et al. (2002) observed decreased birth weights of large-breed foals born to small-breed mares. The difference in the effect of transferring a small-breed embryo to a large-breed dam may be due to species differences. For example, body weight of Cheviot ewes was 77.9% of that of Suffolk ewes (Sharma et al., 2012) while Ponies carrying Pony foals weighed only 50.2% as much as Thoroughbreds carrying Thoroughbred foals (Allen et al., 2002).

Two hypotheses were developed to explain the effect of prenatal environment on development. The thrifty phenotype hypothesis was first presented and examines the nutritional cause of fetal programming, while the predictive adaptive response took a broader approach at rationalizing the programming effects caused by a variety of factors. The thrifty phenotype hypothesis suggests that a fetus receiving poor nutrition will prioritize essential body organs at the expense of others, resulting in a postnatal metabolic profile that takes advantage of limited resources (McMillen and Robinson, 2005). For example, rat fetuses exhibiting intrauterine

growth retardation (IUGR), a result of fetal undernutrition, had reduced glucose transport to lung tissue while maintaining normal glucose transport to the brain (Simmons et al., 1992). These adaptations are homeostatic in nature, making immediate and permanent adjustments to help the body overcome impediments that would otherwise reduce survivability (McMillen and Robinson, 2005). To determine which traits may be considered thrifty, Qasem et al. (2011) restricted maternal protein intake in rats throughout gestation and lactation and measured several variables in their offspring. Compared with pups born to control-fed dams, pups from restricted dams were heavier, hyperphagic, had a greater feed efficiency, and had greater leptin concentrations until day 65 of age. Core body temperature was additionally increased in restricted offspring, indicating an increased metabolic rate. These adaptations are meant to create an individual that is better suited to cope with decreased protein intake throughout life. The authors of this study also reported improved insulin sensitivity in pups of restricted dams, but this is in disagreement with other models of IUGR (Simmons et al., 2001; Rozance et al., 2006). Decreased insulin sensitivity, or insulin resistance, is characterized by an impaired ability of insulin-sensitive tissues to import glucose, resulting in an increase in blood insulin concentration without a concomitant decrease in glucose concentration. According to Barker (2004), insulin resistance may be a thrifty characteristic because, by reserving glucose in the blood, the brain, which has greater priority than other tissues, has greater access to it. However, these studies used fetal hypoglycemia (Rozance et al., 2006) and uterine artery ligation (Simmons et al., 2001), rather than protein restriction to restrict fetal nutrition. It is therefore possible that protein restriction elicits a different set of changes to the intrauterine environment that have a different effect on the programming of insulin sensitivity. Insulin resistance has also been reported in models of developmental programming caused by maternal obesity and overfeeding (Long et al.,

2010; Zhang et al., 2010), which the thrifty phenotype hypothesis fails to explain.

Consequently, the predictive adaptive response hypothesis was developed to create a more universal justification.

According to the predictive adaptive response, the body uses the intrauterine environment as an indication of postnatal conditions and makes adaptations best suited to these forecasted conditions (Armitage et al., 2005). In models of maternal overnutrition, intrauterine conditions signal to the fetus that nutrients will always be plentiful, and development is directed in such a way that creates a phenotype best matched to this scenario. It has additionally been observed that fetal programming may result from fetal hypoxia and stress, and this hypothesis can encompass these causes as well. The predictive adaptive response hypothesis also explains why certain adaptations confer an advantage and others are detrimental. If the prenatal and postnatal environment match, then the individual is well suited to its environment and thrives; on the other hand, if the postnatal environment is different, then the individual is not equipped to handle this set of conditions and suffers a range of consequences (Armitage et al., 2005). In a reduced maternal nutrition model of IUGR, adult rats born to dams on restricted feed during gestation weighed less at birth than offspring of ad libitum fed dams (Tosh et al., 2010). When restricted offspring were suckled by restricted-fed dams, they weighed the same at 9 months as ad libitum offspring suckled on ad libitum-fed dams. However, restricted offspring suckled on ad libitum-fed dams were heavier, had increased IGF-I expression, and had increased IGF-I serum concentrations compared with both other treatments. These rats hadThe negative effects of the altered prenatal environment only emerged when the postnatal environment diverged from the prenatal environment.

## **Causes of Developmental Programming**

The same conditions found in the postnatal environment (nutrient availability, temperature, the presence of toxins or other compounds, or oxygen availability) affect growth in the intrauterine environment. The combined effects of these conditions influence fetal development by changing fetal nutrition or inducing fetal stress.

### **Fetal Nutrition**

Fetal nutrition is critical in directing fetal development. Maternal diet, maternal nutrient stores, nutrient delivery to the placenta, and placental transfer of nutrients to the fetal compartment determine total fetal nutrition, and a deficit in any of these categories can result in impaired fetal nutrition. Maternal under- or overnutrition has been shown to cause changes in fetal development. Blood glucose concentrations decreased in pregnant ewes and in their fetuses following a three-day fast, illustrating the relationship between maternal diet, maternal glucose concentration, and fetal glucose concentration (Oliver et al., 1993). Pregnant multiparous ewes were fed a diet meeting either 100% (control) or 70% (underfed) of daily maintenance requirements and differences in growth of singleton fetuses were observed at day 45, day 90, and day 135 of gestation. Differences observed at day 45 and 90 were limited and did not always carry through to day 135, which may indicate that compensatory gain had occurred in delayed organs.. However, at day 135, underfed fetuses were lighter, and had a smaller thoracic girth and kidney, gut, heart, and pancreas weight (Osgerby et al., 2002). Although not apparent close to term, the brains of underfed fetuses were significantly lighter at day 90 of gestation, which may indicate some deficits in neuronal development. Underfed ewes had decreased circulating glucose concentrations and a lower body condition score than well-fed ewes, indicating less available glucose a smaller reserve for energy mobilization. Differences in placental morphology

were also observed in underfed ewes, as their placentas had significantly more D and C type placentomes and fewer B type placentomes than well-fed ewes (Osgerby et al., 2002). The C and D type placentomes have a greater proportion of fetal tissue to maternal tissue, which has been suggested to develop in poor intrauterine conditions to partition more nutrients to the fetus (Vonhamme et al., 2006; van der Linden et al., 2012). Whereas Vonhamme et al. (2006) reported no differences in vascularity, expression of angiogenic factors, or cell proliferation due to placentome morphology, the effect of placentome type on nutrient delivery has not been studied directly. Based on this evidence, maternal diet may determine fetal nutrition by simultaneously affecting the total substrates available to the fetus and the placental transfer of these substrates.

The primary response of the fetus to reduced nutrition is to slow cellular division (Barker and Clark, 1997). This attempt to preserve what limited nutrients are available may be directly due to a lack of nutrients or a response to changes in hormone or growth factor concentrations (Barker and Clark, 1997). This deceleration in cellular division has been shown to affect cell distribution and organ development. For example, rats that experienced intrauterine growth restriction due to bilateral uterine artery ligation had a 50% smaller pancreatic  $\beta$ -cell mass than control rats (Simmons et al., 2001). If the affected cells include endocrine tissue such as the pancreas, abnormal hormone secretion may ensue, and this can lead to other permanent effects. Reduced cell proliferation can therefore continue to affect lifelong growth if it occurs in endocrine organs that secrete growth-regulating hormones, including the anterior pituitary gland (growth hormone; GH), pancreas (insulin) and liver (insulin-like growth factor I; IGF-I). For example, in 26 week-old growth-restricted rats exhibiting decreased  $\beta$ -cell masses, serum insulin concentration was reduced following a glucose infusion, indicating defects in insulin secretion and action (Simmons et al., 2001). Similarly, in an obese ovine model of developmental programming, fetal



pancreatic  $\beta$ -cell numbers were reduced by late gestation and lambs of obese ewes had decreased circulating insulin and were hyperglycemic at birth (Zhang et al., 2010).

Increased maternal dietary intake during gestation also affects fetal nutrition and may cause developmental programming to occur. Rodent models (Portha et al., 2011) as well as ovine models (Long et al., 2010; Adam et al., 2011; Zhang et al., 2011) have revealed detrimental lifelong effects in the offspring of overfed mothers. At day 130 of gestation, fetuses of ewes fed a high-intake diet from day 35 of gestation were significantly heavier, contained more adipose tissue, and had decreased plasma insulin concentrations (Adam et al., 2011). Maternal overnutrition may therefore lead not only to increased body weight in the offspring but also to a preferential increase in adipose tissue. Fetuses of overfed ewes also had decreased plasma insulin concentrations and alterations to the hypothalamic leptin-signalling pathway. Both insulin and leptin are involved in the regulation of metabolism, so alterations to their function have implications on feed intake (Coupé et al., 2012), nutrient partitioning (Simmons et al., 2001), and tissue development (Thomas et al., 2001; Croze et al., 2012).

Whereas Adam et al. (2011) investigated the effect of early-gestation overnutrition, Long et al. (2010) used an obese overnourished ewe model by overfeeding ewes preconception so they were already overweight by embryo implantation. Ewes were fed either a control (100% NRC recommendations) or overfed (150% NRC recommendations) diet from 60 days before conception until parturition. Lambs were allowed to nurse from the ewe and were subsequently weaned at 120 days of age. There was no effect of maternal overnutrition on offspring body weight from birth until 19 months of age. However, following a 12-week feeding challenge, offspring of overfed ewes showed significant increases in feed consumption, plasma leptin concentration, fasting glucose and insulin concentration, and change in fat mass compared with

lambs born to control-fed ewes. This study supports the theory that maternal condition, in addition to maternal diet, affects the offspring, since these overfed ewes had increased body weights and body condition scores compared with control-fed ewes from conception to parturition. A comparison of the studies by Adam et al. (2011) and Long et al. (2010) helps to illustrate the importance of the timing of the insult during development. Different tissues and organ systems are sensitive to stimuli at different stages of development, and it has been hypothesized that this period of sensitivity may be determined by the period of rapid cellular development for a specific tissue (Barker and Clark, 1997). Long et al. (2010) increased maternal diet beginning 60 days before conception, while Adam et al. (2011) did not introduce the experimental diet until embryo transfer. It may be that only a change in nutrition has a greater effect on fetal growth than a preexisting state of poor nutrition. It is also possible that a compensatory mechanism accounted for the loss of a difference in fetal weight between day 130 and birth (approximately day 147). This hypothesis is supported by the observation that there was no difference in body weights of 135 day-old fetuses from control- or overfed ewes on an identical feeding and breeding regimen to those in the study by Long et al. (Zhang et al., 2011).

Maternal diet may influence fetal nutrition either directly by a lack or excess of nutrients available to the fetus or by impairing placental transfer of these substrates to the fetus. The fetus consistently consumes 10% of maternal glucose production, irrespective of decreased maternal glucose (Hay et al., 1983). If maternal plasma glucose concentration increases or decreases substantially, then the fetus will accordingly acquire an increased or decreased supply of glucose. The placenta partitions nutrients between the maternal and fetal compartments, so any defect in its structure or function will affect nutrient supply to the fetus. Placental transport of amino acids occurs via active transport mechanisms (Oliver et al., 1993). Utero-placental insufficiency has

been implicated in instances of developmental programming in rodent studies (Portha et al., 2011). In these studies, fetuses exhibited decreased blood glucose, amino acid, insulin, IGF-I, and oxygen concentrations. Uterine artery ligation has been used to model utero-placental insufficiency, and fetuses of rats with ligated uterine arteries were lighter at day 20 of pregnancy compared with sham-operated rats (Simmons et al., 1992). Maternal overnutrition or obesity may contribute to utero-placental insufficiency and this may be a mechanism through which overnutrition affects fetal growth. When ewes were fed an obesogenic diet from 60 days pre-conception until necropsy, their fetuses were significantly heavier at day 75 of gestation but not different from their control-fed counterparts at day 135, indicating a deceleration in fetal growth in the second half of gestation (Ma et al., 2010). Number and weight of all (types A, B, C, and D) placentomes were recorded, and cotyledonary (COT) and caruncular (CAR) arteriole number, diameter, and angiogenic growth factor expression were measured in two type A placentomes in each placenta. The most salient of their findings include an increase in COT arteriole diameter in overfed ewes compared with control ewes at day 75 and from day 75 to 135 in control but not overfed ewes. They also observed a decrease in total and average placentome weight in overfed ewes on day 135 compared with control ewes on day 135 and both groups on day 75. Blood flow and nutrient exchange are determined by placental vascularity, which is affected by arteriole diameter and placentome size. Therefore, these changes may represent a delayed compensatory mechanism by the maternal placenta to prevent the influx of excessive nutrients and explain the shift in overfed ewes from accelerated growth in early gestation to decelerated fetal growth in later gestation. This compensation is likely mediated through the altered expression of angiogenic growth factors, as the mRNA abundance of vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2), platelet-derived growth factor

(PDGF), and angiopoietin 1 and 2 (ANG-1,2) were all decreased in overfed ewes compared with control ewes at day 75. The juxtaposition of accelerated and decelerated fetal growth may have opposite effects on the development of various tissues and organs, depending on their window of development, and this may ultimately lead to any the negative consequences of developmental programming.

### **Fetal Stress**

Independent of fetal nutrition, changes to the intrauterine environment may promote developmental programming by inducing fetal stress (Barker, 2004). Fetal hypoxia causes a reduction in cellular division (Barker and Clark, 1997), providing a potential mechanism by which hypoxia and fetal stress causes changes in growth and development. When pregnant rats were chronically exposed to hypoxic environments, their 20-day old fetuses exhibited expression of 70 kd heat shock protein (HSP70) in cardiac tissue and aortic wall nitrotyrosine staining, both of which are indications of oxidative stress and the presence of reactive oxygen species (ROS; Giussani et al., 2012). Chronic fetal hypoxia also significantly increased fetal hematocrit, fetal aortic wall thickness, and alterations to adult myocardial contractility in these offspring. Maternal supplementation of ascorbic acid, an antioxidant that scavenges ROS, alleviated these effects, indicating that oxidative stress is, in fact, the mechanism responsible for programming this cardiac dysfunction.

### **Epigenetics as the Programming Mechanism**

One of the characteristics of developmental programming is the persistence of its effects, implying a permanent mechanism of change that occurs without changes to the genome.

Epigenetic inheritance, a pattern of trait inheritance in which gene expression but not gene sequence is changed in response to extracellular stimuli (Ford and Long, 2012), is the likely mechanism. Gene expression may be altered epigenetically through DNA methylation at the 5'-position of cytosine residues within CpG islands or through post-translational histone modification. The most common mode of epigenetic control in mammals is DNA methylation of cytosine residues of CpG islands (Ford and Long, 2012). Normally, the embryonic genome is demethylated after fertilization, and remethylation begins soon after implantation. Therefore, the intrauterine environment during the peri-implantation period is integral in determining the remethylation patterns of the genome of the conceptus and affecting development (Wu et al., 2004).

Many animal models have supported the role of epigenetics in developmental programming. The majority of these are rodent models, and they have shown epigenetic alterations as a result of maternal nutritional changes and uterine artery ligation in tissues such as the pancreas, skeletal muscle and liver (Park et al., 2008; Raychaudhuri et al., 2008; Thompson et al., 2010; Tosh et al., 2010). Pancreatic  $\beta$ -cell dysfunction is a common consequence of intrauterine growth retardation. Impaired expression of the transcription factor for  $\beta$ -cell development *Pdx1* has been implicated in several cases of metabolic syndrome and was observed in IUGR rat offspring (Park et al., 2008). Fetuses also exhibited deacetylation of histones H3 and H4 of this gene, while  $\beta$ -cells of two week-old pups showed methylation on histone 3 lysine 9 (H3K9). Methylation of a CpG island followed in adult rats. Histone acetylation is associated with a more accessible chromatin fiber and increased gene transcription while histone methylation decreases transcription. Therefore, the epigenetic pattern observed by Park et al. (2008) indicates a progressive silencing of the *Pdx1* gene. Thompson et al. (2010) expanded on these findings by

performing a whole genome cytosine methylation assay on pancreatic islets obtained from 7 week-old IUGR rat offspring. One thousand loci with significantly different methylation patterns between IUGR and control offspring were identified and studied, and these occurred mainly at intergenic sequences. Consequentially, it can be concluded that epigenetic modifications are involved in the development of permanent changes to the pancreatic islet in response to IUGR.

Meanwhile, mRNA expression of insulin-sensitive GLUT4 glucose transporters decreased in skeletal muscle of female adult rats whose dams received reduced nutrition from day 11 to 21 of gestation compared with those born to control dams (Raychaudhuri et al., 2008). Decreased GLUT4 activity is one of the factors contributing to insulin resistance and glucose intolerance, two components of the metabolic syndrome observed in growth-restricted offspring. In this study, several epigenetic control mechanisms were altered, including DNA methyltransferase recruitment, histone acetylation and recruitment of *glut4* promoter inhibitors and activators. Similarly, in the adult liver of IUGR rat offspring fed ad libitum post-natally, mRNA expression was increased in several of the IGF-I regions compared with rats born to control dams (Tosh et al., 2010). These animals also exhibited decreased rates of H3K4 dimethylation at day 1 of age on several of the IGF-I regions. This pattern of dimethylation continued at 9 months of age, at which time point trimethylation of H3K4 increased in restricted offspring fed ad libitum post-natally compared with restricted offspring maintained on a restricted diet. Di- and Trimethylation of the H3K4 locus is a marker of increased activated gene expression. These results provide additional evidence for a change in gene expression in response to IUGR, possibly manifesting as a decrease in IGF-I expression at birth when nutrient supply was low and increasing in early postnatal life when nutrient supply increased. Beyond the studies presented thus far, there have also been reports of changes in methylation states of glucocorticoid receptor, peroxisome

proliferator-activated receptor  $\alpha$  (PPAR- $\alpha$ ), and angiotensin receptor in rat offspring in response to changes in maternal protein intake (Portha et al., 2011). These findings support the theory that epigenetic dysregulation is responsible for translating fetal environment to permanent changes in phenotype.

### **Effects in the Offspring**

The effects of developmental programming seen in the offspring are varied and quite often interrelated. In general, the effects can be divided into growth pattern alterations, dysregulation of metabolism, or organ dysfunction.

### **Growth Pattern Alterations**

Depending on the timing and nature of the insult to the fetus, growth may be retarded or accelerated at various stages during prenatal or postnatal development. Altered postnatal growth can be a main effect of developmental programming, while altered prenatal growth can be a response to intrauterine conditions that will cause other permanent changes in later life.

Intrauterine growth retardation falls into a category that is a blend of cause and effect. It is an effect of reduced maternal nutrition (Tosh et al., 2010) or reduced utero-placental efficiency (Simmons et al., 2001), but it can also cause myriad other lifelong consequences. These consequences include, but are not limited to, impaired hormone regulation, insulin resistance and glucose intolerance, altered body composition, and compensatory growth (Simmons et al., 2001; Osgerby et al., 2002; Ford et al., 2007; Tosh et al., 2010). To understand how IUGR can contribute to these effects, it is necessary to understand the importance of mismatch between prenatal and postnatal environment. Fetal growth is restricted in response to starvation

conditions, and the body is programmed to take advantage of available nutrients as soon as they become present. This conditioning persists into postnatal life, and when the offspring is presented with adequate nutrition, the body is still programmed to efficiently use as much of the substrates as possible, resulting in accelerated growth. This can stress the limits of organs designed to meet a reduced functional need (Barker, 2004), leading to other complications. Compensatory growth can also occur prenatally when maternal nutrition during gestation is restored following a period of restriction (Adam et al., 2011). Lambs whose mothers were fed a restricted diet between day 28 and 78 of gestation weighed the same as lambs born to control-fed mothers at birth, but trended to be heavier by day 8 and were significantly heavier at 4 months of age (Ford et al., 2007). These animals also had greater backfat measurements, greater circulating leptin concentrations, and an impaired insulin response to a glucose tolerance test. Although restricted lambs did not have reduced birth weights, IUGR is still indicated by the development of compensatory growth and other impairments. It is possible that growth was restricted during the mid-gestation period of reduced nutrition and then accelerated throughout the last half of gestation when proper nutrition was restored.

However, these changes can occur without the observation of IUGR, and the timing of the insult may be the most important factor in determining the observed effects. Ford et al. (2007) restricted maternal diet during mid-gestation and observed no effect on birth weight. Meanwhile, Tosh et al. (2010) restricted maternal diet of pregnant rats during mid- and late-gestation and observed reduced birth weights of IUGR newborns. Both studies reported effects on postnatal growth, body composition, and circulating hormones. Intrauterine growth retardation can also occur as a result of maternal overfeeding, and this effect is also sensitive to period of development. Lambs born to obese ewes that were overfed from pre-breeding until parturition



did not differ in birth weight from lambs born to control ewes (Long et al., 2010), while 130-day-old fetuses from ewes fed a high intake diet from day 35 to 130 were significantly lighter than their control counterparts (Adam et al., 2011).

### **Metabolic Dysregulation**

Animals exhibiting developmental programming often have impaired metabolism regulation as indicated by altered circulating hormone concentrations, including insulin, leptin, IGF-I, and IGF binding proteins (IGFBP).

The activity of insulin, an anabolic protein hormone that stimulates glucose-uptake in sensitive tissues and is secreted by  $\beta$ -cells of the pancreas, is influenced by developmental programming. In vitro glucose-stimulated insulin secretion was significantly reduced in islets isolated from ovine fetuses exposed to late-gestation hypoglycemia (Rozance et al., 2009). Isolated islets were found to contain normal insulin stores, so the defect in insulin secretion is most likely due to a defect in insulin exocytosis rather than synthesis. A rat model of utero-placental insufficiency-induced IUGR showed similar results (Simmons et al., 2001). At one week of age, there was no difference in fasted glucose or insulin concentrations between IUGR and control offspring, but fasting hyperglycemia and hyperinsulinemia developed in IUGR offspring at seven weeks of age. Hyperglycemia continued throughout the study, but the hyperinsulinemia regressed after fifteen weeks of age. Glucose tolerance tests and insulin tolerance tests at various time points revealed impaired glucose-stimulated insulin secretion and impaired glucose clearance in response to insulin, respectively. Rozance et al. (2009) concluded that impaired insulin activity is due to a secretory defect, since  $\beta$ -cell numbers remain consistent between hypoglycemic and control fetuses. Simmons et al. (2001) also observed similar  $\beta$ -cell

mass, as well as islet size and pancreatic weight, between treatment groups at week one and seven. However, the relative  $\beta$ -cell mass in 15-week-old IUGR offspring decreased to 50% that of control offspring and further decreased to less than one-third that of control offspring by week 26. Therefore, the insulin resistance and glucose intolerance observed in response to developmental programming may be a result of impaired insulin secretion perinatally and a conjunction of impaired secretion and reduced  $\beta$ -cell mass later in life.

The hormone leptin is secreted by white adipose tissue and regulates metabolism by acting on specific neurons in the brain that control energy balance (Thomas et al., 2001; Coupé et al., 2012). Leptin secretion decreases appetite by binding leptin receptors in sensitive nuclei in the brain, and it also has peripheral effects (Desai et al., 2007). Fetuses necropsied at day 130 of gestation from ewes fed a high intake diet from day 35 of gestation exhibited alterations to the leptin-signaling pathway. There was no difference in circulating leptin concentration between overfed and control fetus treatment groups, but gene expression of anorexigenic neuropeptide (CART) is decreased in the overfed ewe–IUGR fetal group. In addition, in rodents, an early postnatal surge in leptin concentrations is thought to program the activity balance in orexigenic and anorexigenic neurons and leptin sensitivity (Yura et al., 2005). This peak was also observed in lambs, but it was eliminated in lambs born to obese overnourished ewes (Long et al., 2010). Defects in leptin regulation may therefore contribute to the increase in adiposity associated with IUGR and developmental programming.

Insulin-like growth factor-I is one component of the somatotrophic axis, a group of interrelated hormones that regulate growth and metabolism, and which include growth hormone, IGF-I, and the IGFBPs. Two neuropeptides from the hypothalamus act on the anterior pituitary gland to regulate GH secretion; GH releasing hormone (GHRH) stimulates its secretion while

somatostatin (SHRH) is inhibitory. Once released in circulation, GH binds to its receptor (GHR) to stimulate IGF-I release in sensitive tissues and mediate its somatogenic effects through this hormone. There are primarily GHR present in liver, and some have also been identified in adipose tissue (Sørensen et al., 1992). Insulin-like growth factor-I acts in an endocrine fashion when secreted by the liver in response to GH and in an auto- or paracrine manner when secreted by select sensitive tissues. The primary action of IGF-I is to promote cellular growth by stimulating the synthesis of DNA, RNA, and protein (Clemmons and Underwood, 1991). Muscle and bone express IGF-I receptors while adipose tissue does not (Zapf et al., 1978), so increased IGF-I concentration preferentially promotes growth of muscle and bone, resulting in a leaner animal. While IGF-I is secreted in response to the binding of GH to hepatic IGF-I receptors, adequate nutrition is required to increase IGF-I concentrations (Clemmons and Underwood, 1991; Grant et al., 1973). In humans, reduced cord blood IGF-I concentration is associated with preterm growth-restricted fetuses (Chiesa et al., 2008). Runt piglets, which most likely arise due to limited oxygen or nutrient supply, had decreased circulating IGF-I concentrations at birth compared with control piglets (Ritacco et al., 1997). Based on these data, it can be concluded that IGF-I is important for fetal growth and is sensitive to intrauterine conditions associated with developmental programming. Furthermore, liver IGF-I was significantly decreased in newborn IUGR rats compared with control pups, but increased significantly compared with control rats by adulthood (Tosh et al., 2010). The birth- and adult weights of these animals followed the same pattern, so IGF-I may play a role in the mechanism of compensatory growth.

Six IGFBP control the availability of IGF-I (and IGF-II) to tissues, and these are also affected by nutrition (Clemmons and Underwood, 1991). Fetal growth is dependent on IGFBP-2 concentration, as this is the most abundant IGFBP in fetal blood. Approximately 90% of IGF-I in

the blood is bound to IGFBP-3 in a 150 kDa ternary complex that also contains an acid labile unit. When bound to IGFBP-3, the half-life of IGF-I is extended from about 10 minutes to 12 hours, which stabilizes its activity (Kelley et al., 1996). Proper regulation of IGFBP-3 accounts for the constant daily concentration of IGF-I. The activity of IGF-I is also influenced by IGFBP-2 actions, although not to the same extent as IGFBP-3. There have reports of both inhibitory and stimulatory effects of IGFBP-2 on IGF-I. During fetal development, IGFBP-2 is important for pituitary development and it remains as the predominant IGFBP in cerebrospinal fluid through adulthood. Chronic changes in nutrient intake can influence blood concentration of IGFBP-2 and IGFBP-3 (Clemmons and Underwood, 1991). Protein restriction results in increased hepatic IGFBP-2 mRNA expression, whereas protein restriction or prolonged fasting decreases IGFBP-3 concentrations. These patterns have been repeated in models of maternal nutrition-induced developmental programming. When ewes were fed a reduced intake diet from day 26 of gestation until necropsy, 90-day and 135-day old fetuses had altered circulating concentrations of IGFBP-2 and IGFBP-3 (Osgerby et al., 2002). The concentration of IGFBP-2 increased significantly from day 90 to day 135 in fetuses from underfed ewes compared with those from control-fed ewes, while the concentration of IGFBP-3 only trended to decrease in fetuses from underfed ewes. These reports demonstrate that IGFBP-2 and IGFBP-3 respond to changes in nutrition.

There have been reports of changes in GH action in animals exposed to a modified intrauterine environment (Costine et al., 2005; Koch et al., 2010). At birth the expression of hepatic GH receptors was increased in male offspring of ewes treated with sub-cutaneous GH at breeding compared with control lambs (Koch et al., 2010). Hepatic IGF-I expression was similarly increased in these animals. Tissue from ewe lambs were not collected in this study, but

those from the GH group remained heavier than their control counterparts from birth until 100 days of age, indicating an effect on postnatal growth and development. Normally, an infusion of GH releasing hormone (GHRH) results in an increase in GH concentration, which then stimulates IGF-I secretion by the liver. The lambs in this study additionally exhibited an attenuated IGF-I response to a GHRH challenge, further substantiating a change to the GH axis.

Hormone dysregulation is closely related to changes in growth, as concentrations of insulin, leptin, IGF-I, and IGFBP all have implications for fetal and postnatal tissue accumulation. During fetal development, insulin rather than GH stimulates the release of IGF-I, so insulin and IGF-I are therefore both necessary for proper fetal growth (Gluckman and Pinal, 2003). Postnatally, insulin promotes tissue accretion. Leptin concentrations are correlated with body weight, body weight gain, body condition score, and carcass fat percentage (Thomas et al., 2001). Impairment of leptin action can result in an increase in body condition score and carcass fat.

### **Organ Dysfunction**

Non-endocrine tissues can also acquire defects during developmental programming, resulting in organ dysfunction. Livestock models of developmental programming have revealed changes to the gastrointestinal tract, reproductive tract, brain, kidney, cardiovascular system, and immune system (Reynolds et al., 2010). For example, undernutrition of pregnant ewes from day 26 of gestation resulted in a reduction in brain weight in 90-day-old fetuses and in gut, kidney, heart, and pancreas weight in 135-day-old fetuses compared with fetuses from well-nourished ewes (Osgerby et al., 2002). Reductions in organ weight reflect reductions in development, and are specific to window of development of each organ.

## **The Relationship Between Maternal Hormones and Developmental Programming**

The key to understanding how the intrauterine environment induces specific changes in fetal development must lie in understanding the changes that occur within the body of the ewe and how the embryo or fetus interprets these changes. Maternal hormones regulate metabolism, tissue accretion, the availability of nutrients to the fetus, and development of the placenta. Therefore, maternal circulating hormones may have an important direct or indirect effect on fetal development during gestation.

Nutrient availability to the fetus determines its capacity for growth. Maternal blood composition is one determinant of fetal nutrition, especially in terms of glucose concentration. A cascade of events beginning with maternal blood glucose availability affects fetal growth. Fetal blood glucose concentration changes concurrently with alterations in maternal blood glucose concentration (Oliver et al., 1993; Rozance et al., 2006). Fetal blood glucose stimulates the release of fetal insulin. Whereas GH is responsible for IGF-I secretion by the liver during adult life, insulin stimulates the release of IGF-I during fetal life, and IGF-I mediates fetal growth and development (Gluckman and Pinal, 2003). When nutrients are scarce, a decrease in fetal insulin restricts excess growth to conserve limited nutrients. Changes in maternal glucose concentration will lead to changes in fetal glucose concentration, thereby altering the release of insulin and IGF-I and affecting growth. As such, hormones that regulate nutrient availability in the mother play an important role in determining fetal nutrition and development.

Insulin is the primary hormone that regulates glucose availability in the mother. In many of the animal models reporting evidence of developmental programming in response to changes in maternal nutrition, maternal insulin concentration was altered due to changes in plane of

nutrition. Ewes fed a high-intake diet had increased circulating insulin concentrations (Adam et al., 2011), whereas underfed pregnant ewes had decreased circulating insulin concentrations between days 97 and 132 of gestation and decreased glucose concentrations between days 27 and 132 (Osgerby et al., 2002). It was previously thought that the nutrient demands of the fetus took precedence over those of the mother (Barcroft, 1947), so a change in maternal nutrition should not affect fetal nutrition or growth even though the needs of the mother may be sacrificed. However, the present theory accepts that fetal, maternal, and placental demands are integrated so that neither is sacrificed and the survival of both mother and fetus is maximized. In conditions of maternal starvation, uptake of maternal glucose is decreased in both the fetal and uteroplacental tissues, such that the fetus consistently consumes 10% of maternal glucose production (Hay, 1983).

In addition to insulin, concentrations of leptin are sensitive to body composition or nutrient intake. Consequentially, leptin may play a role in determining fetal growth in response to different levels of nutrition. As gestation progresses, circulating leptin concentrations in the ewe decreases (Thomas et al., 2011). This decrease is most likely due to a shift to a catabolic state as fat is mobilized from adipose tissue to meet the growing energy needs of the ewe. Changes to plane of nutrition lead to changes in leptin concentration. Ewes fed a high-intake diet (i.e., total amount in kg/day) have increased concentrations of circulating leptin (Thomas et al., 2001; Adam et al., 2011). Leptin mRNA expression, as well as protein concentration, was increased in perirenal fat as well (Thomas et al., 2001). When switched from a high-intake to a moderate-intake diet or vice versa, pregnant ewes exhibited an immediate change in leptin concentration. This response may indicate either that leptin has some dependency on diet in addition to body fat percentage, or that an interaction with insulin is the cause for the change (Thomas et al., 2001).

In rodents, an early postnatal surge in leptin concentrations is thought to program the activity balance in orexigenic and anorexigenic neurons and leptin sensitivity (Yura et al., 2005). This same peak was observed in lambs, but it was eliminated in lambs born to obese overnourished ewes (Long et al., 2001). It is possible that the elimination of the leptin peak may be the mechanism responsible for the effects of developmental programming seen in offspring of overfed mothers, but it unclear whether this is a result of alterations in maternal leptin concentration or other factors associated with increased body condition.

The somatotrophic axis is additionally affected by maternal nutrition and may mediate fetal growth by regulating metabolism as well as placental development. Prolonged fasting is associated with increased GH concentrations, resulting from a decreased clearance of GH from the blood (Renaville et al., 1992). Ewes treated with GH administration either at breeding or during gestation had heavier lambs at birth or near term than control ewes (Costine et al., 2005; Koch et al., 2010; Adam et al., 2011). Insulin-like growth factor I regulates glucose and amino acid transport across the placenta (Osgerby et al., 2002), so GH mediates placental nutrient transport indirectly via stimulation of IGF-I release (Koch et al., 2010). An infusion of GH increased maternal blood glucose in pregnant ewes in an overfed model of IUGR (Adam et al., 2011), so it can consequentially be inferred that an increase in maternal GH can influence fetal growth by increasing maternal glucose. In addition to affecting fetal nutrient concentration directly, maternal GH affects fetal nutrition through its effect on placental growth. Growth hormone administration is reported to enhance placental villus area in sheep (Harding et al., 1997). This can enhance placental transport, making more nutrients available to the fetus and allowing a greater capacity for fetal growth. In addition, treatment with a sub-cutaneous injection of GH at breeding significantly affected placental growth in these ewes compared with ewes



receiving no GH (Costine et al., 2005). Placental weight of GH-injected ewes significantly decreased at 80 days of gestation but returned to normal near term (140 days). At 80 days of gestation, a lighter chorioallantois is associated with a more efficient absorption of the histotroph, a major source of fetal nutrition at this time (Roberts and Bazer, 1988).

The effect of nutrition on IGF-I concentrations in pregnant sheep is not entirely clear. Concentrations of IGF-I increase throughout gestation, but are not significantly different between control and underfed ewes (Osgerby et al., 2002). However, IGF-I concentrations in underfed ewes tended to be increased compared with control-fed ewes until day 97 of gestation, when they decreased until the end of gestation. However, a 48 h period of maternal starvation significantly reduced maternal circulating IGF-I concentration, suggesting that acute changes in diet can affect IGF-I concentration (Oliver et al., 1993). There was also a decrease in fetal glucose during this period, which strengthens the theory that maternal IGF-I concentrations mediate fetal growth through changes in placental glucose transport. However, maternal plasma insulin also fell during this period, so it is unclear whether one or both of these hormones played a role in the decreased glucose transport, or if the decrease in fetal glucose is solely due to a decrease in maternal glucose. It has been shown that treatment with endogenous IGF-I in the pregnant sheep promotes fetal growth without increasing placental size, signifying an enhancement of placental function (Jenkinson et al., 1999). It is therefore likely that maternal IGF-I influences fetal growth by regulating nutrient transport across the placenta. The relationship between maternal nutrition and IGF-I concentration needs to be studied further, but an IGF-I decrease due to decreased nutrition could restrict placental nutrient transport and consequentially restrict fetal growth.

## **Implications**

It is clear that IUGR is a multi-faceted syndrome, characterized by a range of causes and consequences. In a way that mirrors its many-sided nature, IUGR holds implications for more than one sector. Specifically, a thorough understanding of IUGR will benefit both animal agriculture and human health.

### **Animal Agriculture**

One of the goals of animal agriculture is to efficiently produce high quality meat, characterized by a high protein and low fat content. Seasonal variation in quality of forage on grazing land can result in poor nutrition for pregnant livestock (Ford et al., 2007), resulting in compensatory gain and altered body composition later in life. This may have a greater implication for industries in which adult weight and carcass composition are particularly important. Altered growth patterns in the offspring caused by poor maternal nutrition have been shown to result in poor carcass quality in beef and pigs (Rehfeldt et al, 2006; Funston et al., 2011). An understanding of the causes of IUGR will help producers avoid the negative consequences associated with the syndrome, thereby ensuring efficient production of good quality meat.

### **Human Health**

Developmental programming has been linked to human disease, including obesity, cardiovascular disease, insulin resistance, and diabetes (Wu, et al., 2004; Ford, et al., 2005; Tosh et al., 2010). It is well known that obesity has become more prevalent than ever in recent years, prompting some to refer to it as an epidemic. In the United States, 65% of the population is overweight, and half of those are considered obese (Zhang et al., 2010). Cardiovascular disease is also widespread, and the National Institute of Child Health and Human Development even

cites coronary heart disease as the leading cause of death among men and women (NICHD, 2003). Although obesity seems to be the major health concern facing this country, maternal undernutrition is not absent, and IUGR affects 5% of all U.S.-born infants (Wu et al., 2004). An understanding of the mechanisms contributing to developmental programming will help to reduce its prevalence and thereby reduce the incidence of these diseases.

## Materials and Methods

### **Animals**

All procedures were approved by the University of Connecticut Animal Care and Use Committee.

Multiparous Dorset (n = 25), Shropshire (n = 7), and Southdown (n = 4; N = 36) ewes from the University of Connecticut flock were used in this study. Estrus was synchronized by the placement of a controlled intrauterine drug release (CIDR; Eazi-breed, Pfizer) device containing 300 mg progesterone for 12 days. A single IM injection of prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ ) was administered directly following removal of the CIDR. Ewes were placed in three pens separated by breed with a single ram from each breed. Once confirmed pregnant by ultrasound, ewes were individually penned and randomly assigned to one of three diets (approximately d 31 of gestation). Control (CON) ewes received 100% NRC (1985) requirements for a pregnant ewe carrying twins, whereas restricted (RES) received 60% and overfed (OVER) received 140% requirements. Diets were comprised of a complete pelleted feed and maintained until parturition. Daily feed allotments were divided equally between two feeding times, 0800 h and 1600 h daily. Feed was weighed before feeding and leftover feed was weighed before the next feeding period to determine total intake. Ewes that began to eat their bedding were provided with straw as a source of roughage that would not contribute any nutritional value. A handful of straw was also mixed into the pellets to prevent ewes from gorging themselves. Ewes were provided with free access to water. Ewes were removed from this portion of the study following parturition.

Ewes were weighed weekly and assigned a body condition score (BCS) between 1 and 5 (1 = emaciated, 5 = obese). Blood samples (20 mL) were obtained weekly by jugular venipuncture and immediately divided between two (7 mL each) silicone coated glass BD vacutainers, one (3

mL) vacutainer containing EDTA and one (3 mL) vacutainer containing heparin. Blood was allowed to immediately clot in silicone coated vacutainers at room temperature for 4 to 6 hours, then at 4 to 6 °C overnight. These tubes were then centrifugated at 1,200 x. g. at 4 °C for 30 minutes and serum was immediately pipetted off into two plastic tubes. Vacutainers containing anticoagulant were inverted 6 to 8 times immediately following collection, then placed on ice until centrifugation at 1,200 x. g. at 4 °C for 30 minutes. Plasma was then pipetted from the pellet, and all sample tubes were frozen at -20 °C until hormone analysis.

## **Hormone Analysis**

### **Growth Hormone**

Serum GH concentrations were determined by radioimmunoassay (RIA) as previously described (Kazmer et al., 1992). Lyophilized ovine GH (Purchased from A. F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases, Torrance, CA) was reconstituted and labeled with I<sup>125</sup> (2 mCi in 20 µL; Cat. # Nez033A002MC, Perkin-Elmer, Shelton, CT). Rabbit-anti-ovine antibody (Parlow) was used as the First Antibody in 1:20,000 dilution, and goat-anti-rabbit γ globulin (Cat. # 539845-10KU, Calbiochem, EMD Bioscience) was used as the Second Antibody in 1:40 dilution. All samples were analyzed in two assays and run in duplicate. The complete protocol can be found in the Appendix.

### **Insulin-like growth factor-I**

Serum IGF-I concentrations were determined by RIA as previously described (Richmond and Zinn, 2009) using  $I^{125}$ -IGF-I (25  $\mu$ Ci; Cat. # Nex-2410, Perkin-Elmer). Rabbit-anti-human-IGF-I (Parlow) was used as the First Antibody in 1:100,000 dilution, and goat-anti-rabbit  $\gamma$  globulin (Calbiochem) was used as the Second Antibody in 1:4 dilution. All samples were analyzed between three assays and run in duplicate. The complete protocol can be found in the Appendix.

### **Insulin**

Insulin was analyzed using a colorimetric bovine insulin enzyme-linked immunosorbent assay (ELISA; Cat # 80-INSBO-E01, ALPCO Diagnostics, Salem, NH). Serum samples were analyzed in duplicate and steps were followed as per manufacturers instructions. Samples from six Dorsets that gave birth to twins from each treatment group were analyzed. The OVER group only had four Dorsets giving birth to twins, and two Shropshires that gave birth to twins completed this group.

### **Leptin**

Leptin was determined using a commercial multi-species leptin RIA kit (Cat. # XL-85K, Millipore Corporation, Billerica, MA). Plasma samples were analyzed in duplicate. Plasma:assay buffer ratio was optimized and modified from manufacturers instructions as follows: 300  $\mu$ L sample and 100  $\mu$ L Assay Buffer were added to sample tubes. Samples were chosen for analysis as for insulin.

### **Insulin-like growth factor binding proteins**

Western ligand blot analysis was used for determination of IGFBP concentration as previously described (Govoni et al., 2002). A 12 % resolving and 5 % stacking polyacrylamide gel was prepared for use in a mini-protean II electrophoresis unit (Bio-Rad, Hercules, CA). Each well was loaded with 20  $\mu$ L of prepared sample containing 2  $\mu$ L serum sample, 18  $\mu$ L doubly distilled water and 20  $\mu$ L non-reducing load buffer, and samples were run in duplicate. Bovine serum and recombinant human IGFBP-3 were used as standards. Proteins were electrophoresed through gel at 100 V for 30 minutes and 150 V for 1 hour (until dye reached bottom of gel) then transferred to nitrocellulose membrane in a transfer cell (Bio-Rad) at 45 V for 1.5 hours. Membranes were incubated in  $I^{125}$ -IGF-I overnight in 0.1% Tween 20 in Tris-buffered saline (TBS). Membranes were washed after incubation then exposed to a multi-purpose phosphor screen (Packard Instrument Company, Meriden, CT) for 18 hours. Phosphor screens were imaged with a Cyclone Storage Phosphor Sytesm (Packard) and images were analyzed using OptiQuant acquisition and analysis software (Packard). Proteins were measured as digital light units (DLU)/mm<sup>2</sup> and calculated as a percentage of the signal of the bovine standard. A complete protocol can be found in the Appendix.

### **Data Analysis**

Data were analyzed using ANOVA with SAS Statistical Analysis Software (Cary, NC). All longitudinal data were analyzed using the repeated measures method with Proc Mixed. Matrix structures were chosen between the Compound Symmetry (CS), Variance Components (VC), Autoregressive (AR), Hyun-Feltz (HF), and Toeplitz (TOEP) models by choosing the model

with the least AIC. A  $P$ -value equal to or less than 0.05 was considered significant, and trends were considered as  $0.05 < P < 0.10$ .



## Results

Diet affected average body weight (BW), total gain, and average daily gain (ADG) in pregnant ewes (Table 1, Fig. 1). Bodyweight (BW) increased throughout gestation in each of the 36 ewes in the study. There were no differences in BW due to diet at the onset of treatment ( $P > 0.44$ ). By the end of gestation (wk 20), RES ewes ( $89.3 \pm 3.6$  kg) were lighter ( $P < 0.01$ ) and OVER ewes ( $119.7 \pm 3.6$  kg) were heavier ( $P < 0.1$ ) than CON ewes ( $112.9 \pm 3.6$  kg). Similarly, average daily gain (ADG) was increased ( $P < 0.01$ ) in OVER ewes ( $0.35 \pm 0.01$  kg) and decreased ( $P < 0.01$ ) in RES ewes ( $0.23 \pm 0.01$  kg) compared with CON ewes ( $0.27 \pm 0.01$  kg). Average BW throughout gestation was reduced ( $P < 0.05$ ) in RES ewes ( $91.4 \pm 2.9$  kg) compared with CON ewes ( $99.7 \pm 2.9$  kg). However, despite the increase in late gestation BW and ADG, there was no difference ( $P < 0.4$ ) between average BW of OVER ( $103.3 \pm 2.9$ ) and CON ewes.

Average GH concentration increased from wk 8 to 20 in CON and RES but not OVER treatment groups (Table 3). There was no difference between treatment groups until wk 14 ( $P < 0.05$ ), at which point GH concentration increased in RES ewes compared with CON ewes. There was an overall effect of diet on GH concentration in RES ewes ( $6.4 \pm 0.6$  ng/mL), which had greater ( $P < 0.01$ ) concentrations than CON ewes ( $3.2 \pm 0.6$  ng/mL) and OVER ewes ( $2.3 \pm 0.6$  ng/mL) over the duration of gestation (Table 2, Fig. 2A). There was no effect of diet on GH concentration in OVER ewes.

Throughout gestation (wk 8 to 20) average concentrations of IGF-I followed a trend to be greater ( $P < 0.1$ ) in OVER ewes ( $197.7 \pm 14.5$  ng/mL) compared with CON ( $163 \pm 14.5$  ng/mL)

and RES ( $146.35 \pm 14.5$  ng/mL) ewes, but there was no difference between CON and RES ewes ( $P < 0.4$ ; Table 2, Fig. 2B). Concentrations of IGF-I did not differ until after approximately wk 17 of gestation. Overfeeding ewes during gestation resulted in a net increase ( $60.2 \pm 32.9$  ng/mL) in IGF-I concentration, which was not different from the increase observed in CON ewes ( $52.7 \pm 32.9$  ng/mL). In contrast, RES ewes exhibited a net decrease in IGF-I concentration ( $-34.9 \pm 34.3$  ng/mL), which was different ( $P < 0.07$ ) from the net change in CON ewes.

Concentrations of IGFBP-2 but not -3 were sensitive to changes in diet during gestation (Table 2, Fig. 2C, D). Overfeeding ewes resulted in decreased ( $P < 0.01$ ) IGFBP-2 concentration ( $0.53 \pm 0.12$  AU) compared with CON ewes ( $0.97 \pm 0.10$  AU). There was no effect ( $P < 0.7$ ) of underfeeding ewes on IGFBP-2 concentration ( $0.93 \pm 0.06$  AU). Average IGFBP-3 concentrations were  $1.81 \pm 0.14$ ,  $1.64 \pm 0.10$ , and  $1.99 \pm 0.14$  AU in CON, RES, and OVER ewes, respectively, and there were no associated treatment effects ( $P > 0.3$ ).

Insulin increased throughout gestation in all treatment groups (Fig. 2E). Average concentrations of insulin were greater ( $P < 0.001$ ) in OVER ewes ( $0.811 \pm 0.06$  ng/mL) than CON ewes ( $0.396 \pm 0.6$  ng/mL; Table 2). There was no effect ( $P < 0.9$ ) of maternal diet on insulin concentration in RES ewes ( $0.381 \pm 0.06$  ng/mL).

Average concentrations of leptin were greater ( $P < 0.02$ ) in OVER ewes ( $4.3 \pm 0.6$  ng/mL) than CON ewes ( $2.3 \pm 0.6$  ng/mL; Table 2, Fig. 2F). Leptin averaged  $1.5 \pm 0.6$  ng/mL in RES ewes and was not different ( $P < 0.3$ ) from CON ewes.

**Table 1.** The effect of feeding a control (CON), restricted (RES) or overfed (OVER) diet on average body weight (BW; kg), total BW gain (kg), and average daily gain (ADG; kg) in ewes from wk 8 to 20 of gestation. Values are means  $\pm$  SEM.

Treatment	Avg BW (kg)	<i>P</i> -value compared with CON	Total gain (kg)	<i>P</i> -value compared with CON	ADG (kg)	<i>P</i> -value compared with CON
CON	99.7 $\pm$ 2.9		22.8 $\pm$ 1.1		0.27 $\pm$ 0.01	
RES	91.4 $\pm$ 2.9	<i>P</i> < 0.05	10.3 $\pm$ 1.1	<i>P</i> < 0.001	0.23 $\pm$ 0.01	<i>P</i> < 0.01
OVER	103.3 $\pm$ 2.9	<i>P</i> < 0.4	29.3 $\pm$ 1.1	<i>P</i> < 0.001	0.35 $\pm$ 0.01	<i>P</i> < 0.01

**Table 2.** The effect of feeding a control (CON), restricted (RES) or overfed (OVER) diet on concentrations (ng/mL) of metabolic hormones, including GH, IGF-I, insulin, leptin, IGFBP-2, and IGFBP-3, from wk 8 to 20 of gestation. Values are means  $\pm$  SEM.

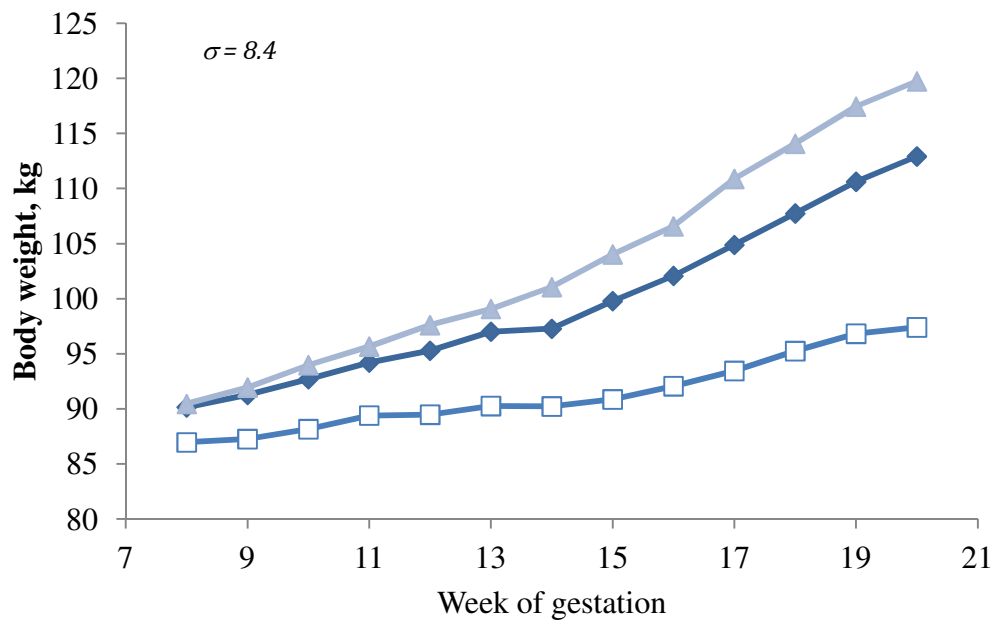
Hormone	Treatment	Avg concentration (ng/ml)	<i>P</i> -value compared with CON
GH	CON	3.15 $\pm$ 0.56	
	RES	6.43 $\pm$ 0.56	<i>P</i> < 0.001
	OVER	2.26 $\pm$ 0.56	<i>P</i> < 0.3
IGF-I	CON	163.02 $\pm$ 14.52	
	RES	146.35 $\pm$ 14.57	<i>P</i> < 0.4
	OVER	197.69 $\pm$ 14.50	<i>P</i> < 0.1
IGFBP-2	CON	0.975 $\pm$ 0.10	
	RES	0.932 $\pm$ 0.12	<i>P</i> < 0.7
	OVER	0.533 $\pm$ 0.06	<i>P</i> < 0.01
IGFBP-3	CON	1.81 $\pm$ 0.14	
	RES	1.64 $\pm$ 0.1	<i>P</i> < 0.3
	OVER	1.99 $\pm$ 0.14	<i>P</i> < 0.3
Insulin	CON	0.316 $\pm$ 0.07	
	RES	0.309 $\pm$ 0.07	<i>P</i> < 0.95
	OVER	0.613 $\pm$ 0.07	<i>P</i> < 0.005
Leptin	CON	2.3 $\pm$ 0.6	
	RES	1.5 $\pm$ 0.6	<i>P</i> < 0.3
	OVER	4.3 $\pm$ 0.6	<i>P</i> < 0.02

**Table 3.** The effect of feeding a control (CON), restricted (RES) or overfed (OVER) diet on the equations of linear regression line in hormones following a linear trend (i.e. GH and leptin).\*

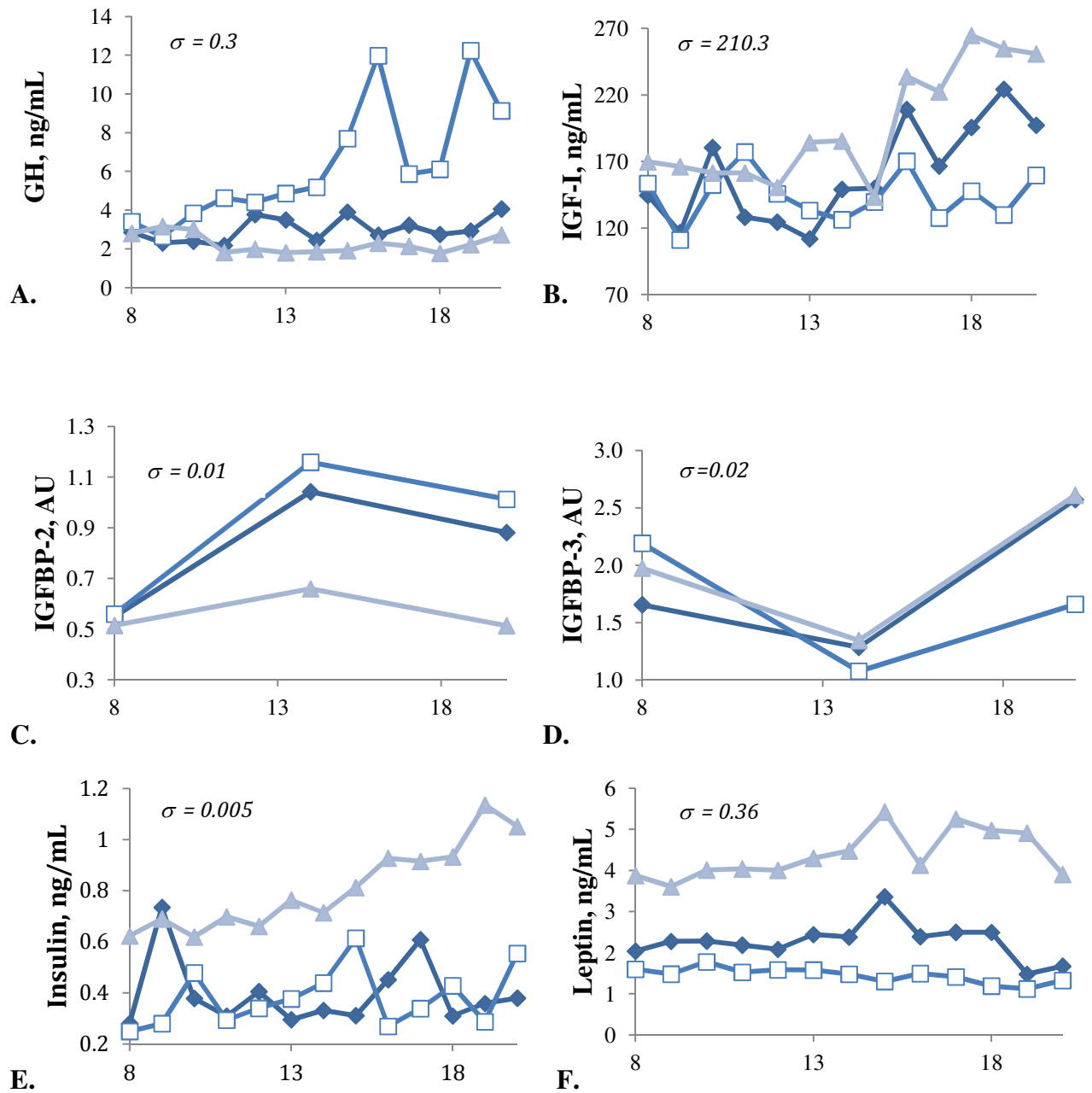
Hormone	Treatment	Equation	R <sup>2</sup>
GH	CON	$y = 0.14x + 1.27$	0.05
	RES	$y = 0.65x - 2.7$	0.17
	OVER	$y = -0.05x + 2.9$	0.01
Leptin	CON	$y = -0.02x + 2.5$	0.02
	RES	$y = -0.04x + 2.0$	0.6
	OVER	$y = 0.09x + 3.2$	0.3

\*  $y$  = hormone concentration

$x$  = week of gestation



**Figure 1.** The effect of feeding a control (CON; ♦), restricted (RES; □), or overfed (OVER; ▲) diet on body weight (kg) in ewes from wk 8 to 20 of gestation.



**Figure 2.** The effect of feeding a control (CON;  $\blacklozenge$ ), restricted, (RES;  $\square$ ), or overfed (OVER;  $\blacktriangle$ ) diet on average concentrations (ng/mL) of GH (A), IGF-I (B), IGFBP-2 (C), IGFBP-3 (D), insulin (E), and leptin (F), from wk 8 to 20 of gestation (x-axis).

## Discussion

Overall, poor maternal nutrition had an effect on the metabolism of pregnant ewes.

Underfeeding ewes resulted in decreased BW gain and increased GH concentration, while overfeeding ewes led to increased BW gain and insulin, IGF-I, and IGFBP-2 concentration.

There were no differences in ewe BW at the beginning of the study, with CON, RES, and OVER ewes averaging  $90.2 \pm 2.9$ ,  $87.0 \pm 2.9$ , and  $90.5 \pm 2.9$  kg, respectively. Underfeeding pregnant ewes decreased their final BW at parturition, while overfeeding increased final BW. Underfeeding had a greater effect than overfeeding, as RES ewes gained  $12.5 \pm 1.1$  kg less BW than CON ewes throughout gestation, but OVER ewes only gained  $6.5 \pm 1.1$  kg more BW than CON ewes. Feeding 140% NRC requirements may not have been enough to elicit a significant change in metabolism. Long et al. (2010) fed ewes 150% NRC requirements from 60d prior conception, and a significant increase was attained within 60 days. However, the current study used an early to late gestation model of poor nutrition rather than a preconception model, and some compensatory mechanisms may be in place after conception that reduce the magnitude of changes resulting from overfeeding. Many studies utilizing ovine models of fetal programming focus on fetal/offspring data rather than maternal BW data (Thomas et al., 2001; Osgerby et al., 2002; Adam et al., 2001), making it difficult to compare the effects of various nutritional models. However, as research in this field continues, it will be necessary to develop clearly defined models of fetal programming.

It is important to note that while there is no difference in BW at wk 8, there is an increase in insulin and leptin concentrations in OVER ewes. Ewes began their diet treatments based on an estimate of gestational age from ultrasound, but their true gestational age was later calculated back from their date of parturition. As a result, some ewes began treatment as early as week 3

and as late as week 8 of gestation. To avoid a small sample size, samples were only analyzed beginning at week 8, when all the ewes had begun their diets and samples were available from each ewe. It is therefore apparent that some of the OVER ewes had been on their increased diet before week 8, and this had a moderate effect on insulin and leptin concentrations. Since there was no concomitant increase in change in BW at week 8, which suggests that insulin and leptin increased in response to a factor independent of BW change. Ruminant pancreatic insulin secretion is influenced by a variety of mechanisms, including blood volatile fatty acid (VFA) content, physiological state, and subtle stimulatory signals (Harmon, 1992). Leptin concentration is proportional to adipose content, so the increase at week 8 may mean that OVER ewes are beginning to gain adiposity quickly after the onset of the diet treatment.

From wk 10 until parturition, RES ewes had greater GH concentrations than CON ewes, in agreement with previous studies that have reported increased GH in fasting ewes (Renaville et al., 1992). Growth hormone promotes placental growth, and while placental development was not evaluated in the present study, this may have been a potential effect of increased GH in RES ewes. Enhanced placental development via augmented GH secretion may be an attempt to compensate for a lack of nutrients and prevent disturbances to fetal development. However, decreased fetal or birth weights most often result from maternal undernutrition (Simmons et al., 2001; Osgerby et al., 2002; Coupé et al., 2012), so this mechanism is not able to overcome the nutrient deficit.

There was no difference in insulin concentration between RES and CON ewes. During times of reduced nutrient availability, glucose uptake is limited in the maternal and uteroplacental tissues to maintain a constant supply (10% of maternal glucose production) to the fetus (Hay, 1983). The reduction change in BW in RES ewes supports the assumption that this was the case



in the present study. Since RES ewes showed no change in insulin in circulation, it is likely that a decrease in glucose uptake is mediated by a decreased in insulin receptor number or affinity in maternal and uteroplacental tissues.

Despite a meager BW change due to overfeeding, overfed ewes showed differences in insulin, IGF-I, and leptin metabolism. The increase in insulin concentration is substantial, with the average concentration in OVER ewes ( $0.811 \pm 0.06$  ng/mL) more than twice the average concentration of CON ewes ( $0.396 \pm 0.145$  ng/mL). These findings are in agreement with those by Zhang et al. (2011), who overfed ewes from 60 d preconception until necropsy at day 135 (approximately wk 19) of gestation. At day 135, overfed ewes had an insulin concentration of  $1.4 \pm 0.05$  ng/mL, which was double that of control ewes ( $0.7 \pm 0.5$  ng/mL). However, in the present study, feeding from wk 5 of gestation on resulted in a three-fold increase in insulin ( $1.14 \pm 0.13$  ng/mL in OVER compared with  $0.36 \pm 0.13$  ng/mL in CON ewes) at the same time point (wk 19). Zhang et al. (2011) supplied overfed ewes with 150% NRC requirements and attained a significant difference in BW by conception that was maintained throughout gestation, and still did not attain such an increase in insulin concentration. A possible explanation is that overfeeding so early allowed the ewes time to adjust to the increased plane of nutrition. Meanwhile, the combination of a suddenly increased intake with an increased nutrient demand by the placental compartment in the present study may have induced the development of insulin resistance in OVER ewes. If increased insulin concentrations in OVER ewes resulted in reduced circulating glucose, then the fetus may receive limited nutrition and thereby experience the effects of fetal programming. On the other hand, if the ewe is experiencing insulin resistance and glucose is not being transported to maternal tissues, then the fetus may in fact receive an overabundance of nutrients. It will be necessary in the future to evaluate the state of glucose

metabolism to determine which of these processes is occurring and at which point in fetal development.

In sheep, leptin concentration has been shown to be related to nutrition, BW, and adiposity (Thomas et al., 2001; Redmer et al., 2012). Increased average leptin concentration in OVER ewes could be caused by any or all three of these factors. Leptin concentration follows a negative trend in CON and RES ewes, but a positive trend in OVER ewes. Therefore, if leptin is an indicator of the metabolic state of the ewe, then it can be concluded that OVER ewes differ from CON and RES ewes in that they are in a catabolic state instead of an anabolic one. Increased leptin in OVER ewes may have several consequences for the offspring. Leptin receptor has been identified in the ovine placenta (Thomas et al., 2001), suggesting that leptin has a role in placental development, and this may be one mechanism by which overfeeding influences fetal development.

Circulating concentrations of IGF-I are sensitive to plane of nutrition, so it is intuitive that OVER ewes have increased ( $P < 0.1$ ) IGF-I concentrations. We also expected that RES ewes would exhibit decreased IGF-I concentrations, but this was not the case. Similarly, Osgerby et al. (2002) reported increasing IGF-I concentrations throughout gestation in restricted and well-fed ewes. Since IGF-I responds to chronic changes in nutrition, restricting feed for this time frame may not have been long enough to elicit a change. Maternal IGF-I affects fetal glucose concentration, likely by a direct effect on transplacental nutrient transport (Jenkinson et al., 1999). Concentrations of IGF-I in OVER ewes were not different from CON ewes until late gestation (after wk 17). This period of gestation is marked by significant fetal growth and limited placental growth (Vonnahme et al., 2006). Therefore, lambs born to OVER ewes have most likely experienced an influx of glucose during this period of rapid development and will exhibit

alterations in tissue accretion and organ development in later life. Indeed, offspring of ewes overfed throughout gestation had greater 130-day fetal weights (Adam et al., 2011) and reduced kidney and pancreas as a percentage of body weight (Zhang et al., 2011).

The increase in IGF-I in OVER ewes may be augmented by the activity of IGFBP-2. In the present study, IGFBP-2 concentration was decreased in OVER ewes, which is consistent in human studies investigating the association between dietary intake and IGFBP concentration (Allen et al., 2002; Crowe et al., 2009). In vitro studies have suggested that one of the roles of IGFBP-2 is to inhibit the activity of IGF-I (Jones et al., 1995). The decrease in IGFBP-2 in OVER ewes would therefore permit an increase in the availability of IGF-I.

There was no effect of overfeeding ewes on IGFBP-3 concentration. These findings are in agreement with those by Crowe et al. (2009), who found no associations between IGFBP-3 concentration and dietary intake.

Woodall et al. (1996) reported increased circulating concentrations of IGFBP-2 and 3 in underfed pregnant Wistar rats compared with their well-fed counterparts. Both IGFBP-2 and 3 have inhibitory effects on the activity of IGF-I, so this increase may help reserve limited nutrients by restricting IGF-I-stimulated growth. However, in the present study, underfeeding pregnant ewes had no effect on either IGFBP-2 or -3 concentration. This may be due to species differences or differences in the level of feed restriction.

Carr et al. (1995) measured IGFBP-3 and IGFBP-2 in the ovine fetus throughout gestation. Fetal concentrations of IGFBP-2 increased between early and mid-gestation and declined from mid to late gestation. This mirrors the pattern of IGFBP-2 concentration in ewes of the present study, which may suggest that maternal IGFBP-2 concentration has a direct effect on fetal

IGFBP-2 concentration. There was no such correlation between maternal and fetal IGFBP-3 concentrations.

In conclusion, poor maternal nutrition alters circulating concentrations of GH, IGF-I, leptin, and insulin, and these changes may mediate the effects of fetal programming seen in offspring. In underfed animals, placental changes mediated by increased GH are most likely the primary hormonal mediator of fetal programming. In overfed animals, leptin also has effects on the placenta, and elevated IGF-I promotes fetal growth due to enhanced nutrient transport. The role of increased insulin in overfed animals in fetal programming will remain unclear until the relationship between insulin and glucose in the ewes can be determined.

## Appendix

### **GH RIA Protocol**

#### Solutions:

##### *1M Dibasic phosphate buffer*

<b>Compound</b>	<b>Amount</b>
Dibasic NaH <sub>2</sub> PO <sub>4</sub> , anhydrous (FW 141.96 g)	141.96 g
dH <sub>2</sub> O	1.0 L

##### *1M Monobasic phosphate buffer*

<b>Compound</b>	<b>Amount</b>
Monobasic NaH <sub>2</sub> PO <sub>4</sub> , anhydrous (FW 119.96)	119.96 g
dH <sub>2</sub> O	1.0 L

##### *0.5 M Phosphate Buffer, pH 7.4*

- Combine monobasic and dibasic phosphate buffers until pH reaches 7.4 and volume is approximately 1.0 L. Previously this was achieved by combining 150 mL Monobasic with 850 mL Dibasic for a total volume of 1 L.

##### *Assay Buffer 0.1% BSA, pH 7.4*

Compound	Amount
0.5 M Phosphate Buffer	100.0 mL
Bovine serum albumin (BSA)	1.0 g
NaCl	8.9 g
dH <sub>2</sub> O	qs to 1.0 L

*First Antibody Buffer*

Compound	Amount
Normal rabbit serum (NRS)	0.25 mL
EDTA	1.86 g
Assay Buffer 0.1% BSA	qs to 100 mL

*First Antibody (1:20,000)*

- Rabbit anti-ovine GH, lyophilized
- A. F. Parlow, NIDDK, Torrance, CA

1. Reconstitute lyophilized antibody with dH<sub>2</sub>O
2. Add 400 µL antibody to 80 mL First Antibody Buffer to attain 1:20,000 dilution

*Second Antibody Buffer, pH 7.4*

Compound	Amount
0.5 M Phosphate Buffer	100 mL

EDTA	18.6 g
PEG 8000 (Carbowax)	50 g
dH <sub>2</sub> O	qs to 1.0 L

### *Second Antibody (1:40)*

- Goat-anti-rabbit  $\gamma$ -globulin
- A. F. Parlow, NIDDK, Torrance, CA
- Contains 125 units i.e. 12,500  $\mu$ L  $\gamma$ -globulin reconstituted
- 0.5 unit (50  $\mu$ L) antibody will precipitate the  $\gamma$ -globulin in 100  $\mu$ L of 2% normal rabbit serum (NRS)

1. Reconstitute lyophilized goat-anti-rabbit  $\gamma$  globulin with 12.5 mL ddH<sub>2</sub>O (no dilution)
2. Add 1 mL antibody to 39 mL Secondary Antibody Buffer to attain 1:40 dilution

### *GH Standard*

- Ovine GH, lyophilized
- A. F. Parlow, NIDDK

1. Reconstitute lyophilized ovine IGF-I to 40 ng/ $\mu$ L

### Day 1:

1. Prepare standard curve
  - a. 8 point curve

- b. Quadruplicates of each point
  - c. Beginning with 40 ng/mL stock solution, serially dilute to create curve:
    - i. 0.1 ng/mL, 0.25 ng/mL, 0.5 ng/mL, 1 ng/mL, 2.5 ng/mL, 5 ng/mL, 10 ng/mL, 20 ng/mL
2. Add 200  $\mu$ L sample, pool, or standard to glass tubes
  3. Add 300  $\mu$ L Assay Buffer to standard, control, and sample tubes
  4. Add 500  $\mu$ L Assay Buffer to NSB and TB tubes
  5. Add 100  $\mu$ L First Antibody Buffer to NSB tubes
  6. Add 100  $\mu$ L I<sup>125</sup>-GH to all tubes (20,000 cpm per tube)
  7. Add 100  $\mu$ L First Antibody to all tubes except NSB and TC
  8. Vortex tubes 1-2 minutes
  9. Cover tubes with tin foil and incubate at room temperature for 24 hours

Day 2:

1. Prepare Second Antibody
2. Add 400  $\mu$ L Second Antibody into all tubes except TC
  - a. Stagger this step so that tubes can be centrifuged immediately after incubation period (i.e. if centrifuge only holds 100 tubes add antibody in groups of 100 tubes)
3. Vortex tubes 1-2 minutes
4. Incubate at room temperature for 1 hour
5. Centrifuge tubes (except TC) at 3,000 rpm for 30 minutes
6. Decant tubes (except TC) and allow to dry upside down overnight on absorbent paper



### Day 3:

1. Count radioactivity on gamma counter (Perkin-Elmer)

### Calculations

1. Calculate average CPM for TC, NSB, TB, standards, pools, and samples
2. Calculate percent binding for TB, standards, pools, and samples
  - a.  $\% \text{ binding} = (\text{CPM} - \text{CPM}_{\text{NSB}}) / (\text{CPM}_{\text{TC}} - \text{CPM}_{\text{NSB}})$
  - b. Total binding (TB) should be over 15%
3. Construct standard curve using % binding for standards
  - a. Plot  $\log(\text{ng/mL})$  v. % binding
  - b. Perform linear regression to obtain standard equation ( $\text{CPM} = a \cdot \log(\text{ng/mL}) + b$ )
4. Calculate ng/tube from standard equation
5. Calculate ng/mL by multiplying ng/tube by dilution factor (5)

## **IGF-I RIA Protocol**

### Solutions:

#### *Assay Buffer, pH 7.5*

<b>Compound</b>	<b>Amount</b>
Tris HCl	14.04 g
Tris Base	1.34 g
Tween 20	2.0 mL
Disodium EDTA	7.4 g
Phenol Red	0.04 g
ddH <sub>2</sub> O	qs to 2 L

- 1) Mix all reagents except Tween 20 and filter using Corning filter into 2 L Pyrex bottle.
- 2) Add Tween 20 and set pH to 7.5.
- 3) De-gas buffer overnight.

*\*\*After buffer has been de-gased, be very cautious when handling it so as to not reincorporate air bubbles into the mixture. Pour very slowly into containers, and never use a funnel.*

#### *Assay Buffer (0.25% BSA)*

<b>Compound</b>	<b>Amount</b>
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Assay Buffer (No BSA) , pH 7.5	1 L
BSA (RIA grade)	2.5 g

- 1) Pour buffer into beaker, then add BSA (so it doesn't stick to glass).
- 2) Allow BSA to dissolve, then mix. (Do not spin stir bar too fast or else it will create bubbles)

*Extraction Buffer (0.05% BSA)*

Compound	Amount
Assay Buffer (No BSA), pH 7.5	2 L
BSA (RIA grade)	1.0 g

- Mix as described for 0.25% BSA Assay Buffer

*Carbowax Solution*

Compound	Amount
PEG-8000 (Carbowax)	8.56 g
Assay Buffer (No BSA)	qs to 200 mL

*0.2M Glycylglycine, pH 2.1*

Compound	Amount
Glycylglycine HCl	3.372 g

ddH <sub>2</sub> O	100 mL
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### *Carrier Solution*

- Normal Rabbit Serum (NRS), lyophilized solid
  - Cat. # 869019, Calbiochem, La Jolla, CA
1. Reconstitute to 5 mL with ddH<sub>2</sub>O
  2. Add 500 µL NRS to 49.5 mL Assay Buffer to attain 1:100 dilution

### *First Antibody (1:100,000)*

- Rabbit-anti-human-IGF-I, lyophilized
  - A. F. Parlow, NIDDK
1. Reconstitute with 500 µL dH<sub>2</sub>O to make a 1:10 dilution
  2. Prepare 1:100,000 dilution
    - a. Add 500 µL reconstituted antibody to 2.0 mL Assay Buffer to make a 1:50 dilution
      - i. These were stored in aliquots in -80 °C freezer
    - b. Add 25 µL (1:50) antibody to 49.975 mL Assay Buffer
    - c. pH should be similar to Assay Buffer (7.5)

### *Second Antibody (1:4)*

- Goat-anti-rabbit γ-globulin, A. F. Parlow, NIDDK

- Contains 125 units i.e. 12,500  $\mu\text{L}$  antibody reconstituted
- 1 unit (100  $\mu\text{L}$ ) antibody will precipitate the  $\gamma$ -globulin in 200  $\mu\text{L}$  of 2% normal rabbit serum (NRS)

1. Reconstitute lyophilized goat-anti-rabbit  $\gamma$  globulin with 12.5 mL ddH<sub>2</sub>O
2. Add 5 mL reconstituted Second Antibody to 15 mL Carbowax Solution to attain a 1:4 dilution

#### *IGF-I Standard*

- Ovine IGF-I, lyophilized
  - A. F. Parlow, NIDDK
2. Reconstitute lyophilized ovine IGF-I to 200 ng/ $\mu\text{L}$
  3. Bring to 5 ng/mL in Assay Buffer (0.25% BSA)

#### *$I^{125}$ -IGF-I*

- Final activity 10,000 cpm/100  $\mu\text{L}$
  - Received lyophilized 25  $\mu\text{Ci}$
  - Cat. # Nex-2410, Perkin Elmer, Shelton, CT
1. Reconstitute lyophilized with 500  $\mu\text{L}$  dH<sub>2</sub>O
  2. Count 5  $\mu\text{L}$  to determine cpm/ $\mu\text{L}$
  3. Add Assay Buffer (0.25%) until activity is 10,000 cpm per 100  $\mu\text{L}$

- i. 8 point curve containing quadruplicates of 10 ng/mL, 20 ng/mL, 40 ng/mL, 80 ng/mL, 160 ng/mL, 320 ng/mL, 640 ng/mL, and 1280 ng/mL

Day 1:

1. Pipette 100  $\mu$ L sample and pools into plastic tube
2. Pipette 100  $\mu$ L Glycylglycine into each tube
3. Vortex 1-2 minutes
4. Cap tubes
5. Incubate at room temperature 36 hours

Day 3:

1. Extract samples

*The final result will consist of 25  $\mu$ L extracted sample and 175  $\mu$ L Assay Buffer (0.25%). This is accomplished using a Digiflex CX pipetting station (Titertek Instruments, Huntsville, AL).*

- a. Add 1 mL Extraction Buffer to sample
    - i. Vortex
    - ii. Repeat twice
    - iii. *With each addition of buffer sample should turn yellow to orange to pink*
  - b. Using pipetting station, add 25  $\mu$ L of sample to 2 tubes, followed by 175  $\mu$ L Assay Buffer (0.25%)
    - i. *Do this quickly so that IGFBP do not re-anneal to IGF-I*
2. Set up assay

- a. Prepare standard curve
  - i. Beginning with 1280 ng/mL stock solution, serially dilute to create curve:
    1. 10 ng/mL, 20 ng/mL, 40 ng/mL, 80 ng/mL, 160 ng/mL, 320 ng/mL, 640 ng/mL, and 1280 ng/mL
    2. Add 200  $\mu$ L each standard to tubes in quadruplicate
  - b. Add 300  $\mu$ L Assay Buffer (0.25%) to NSB tubes
  - c. Add 200  $\mu$ L Assay Buffer (0.25%) to TB tubes
  - d. Add 100  $\mu$ L I<sup>125</sup>-IGF-I into all tubes
3. Vortex tubes 1-2 minutes
4. Cover tubes with tin foil and incubate at room temperature for 24 hours

Day 4:

1. Add 100  $\mu$ L I<sup>125</sup>-IGF-I to each tube
2. Vortex 1-2 minutes
3. Cover with tin foil and incubate at 4 °C for 48 hours

Day 6:

1. Add 400  $\mu$ L Second Antibody to all tubes except TC (no need to stagger)
  - a. Vortex tubes 1-2 minutes
  - b. Incubate at 4 °C for 1 hour
2. Add 100  $\mu$ L Carrier Solution to all tubes except TC
  - a. Vortex 1-2 minutes
  - b. Incubate at 4 °C for 1 hour
3. Centrifuge tubes (except TC) at 4,000 rpm for 30 minutes

- a. Centrifuge in groups of tubes that can be aspirated in less than 5 minutes
4. Aspirate liquid from pellet, being sure not to disrupt pellet
5. Dry uncovered overnight
6. Count radioactivity on gamma counter (Perkin-Elmer)

### Calculations

1. Calculate average CPM for TC, NSB, TB, standards, pools, and samples
2. Calculate percent binding for TB, standards, pools, and samples
  - a.  $\% \text{ binding} = (\text{CPM} - \text{CPM}_{\text{NSB}}) / (\text{CPM}_{\text{TC}} - \text{CPM}_{\text{NSB}})$
  - b. Total binding (TB) should be over 15%
3. Construct standard curve using % binding for standards
  - a. Plot  $\log(\text{ng/mL})$  v. % binding
  - b. Perform linear regression to obtain standard equation ( $\text{CPM} = a \cdot \log(\text{ng/mL}) + b$ )
1. Calculate ng/mL from standard equation



### **IGFBP Western ligand blot**

#### **Solutions:**

##### *Resolving Gel Buffer (1.5 M Tris + 0.4% SDS) pH 8.8*

<b>Compound</b>	<b>Amount</b>
Tris (MW 121.12 g)	90.855 g
Sodium dodecyl sulfate (SDS)	2.0 g
ddH <sub>2</sub> O	qs to 500 mL

##### *Stacking Gel Buffer*

<b>Compound</b>	<b>Amount</b>
Tris (MW 121.12 g)	30.285 g
SDS	2.0 g
ddH <sub>2</sub> O	qs to 500 mL

##### *Acrylamide 30 % (Neurotoxin)*

<b>Compound</b>	<b>Amount</b>
Acrylamide	60.0 g
bis Acrylamide	1.6 g
ddH <sub>2</sub> O	qs to 200 mL

- Dissolve first in 100 mL dd H<sub>2</sub>O, then qs to 200 mL
- Filter after preparing solution
- Store at 4 °C in dark jar

*Persulfate 14%*

Compound	Amount
Persulfate	0.07 g
ddH <sub>2</sub> O	500 µL

- Newly made persulfate lasts about 8 hours

*Non-reducing Load Buffer*

Compound	Amount
SDS	0.8 g
Glycerol	4.0 mL
Bromophenol blue	<i>Enough to color solution</i>
ddH <sub>2</sub> O	qs to 20 mL

*Tank Buffer*

Compound	Amount
Glycine	14.4 g
Tris Base	3.0 g

SDS	1.0 g
ddH <sub>2</sub> O	qs to 1.0 L

*Towbin Buffer*

Compound	Amount
Glycine	28.83 g
Tris Base	6.06 g
SDS	0.6 g
Methanol	400 mL
ddH <sub>2</sub> O	1600 mL

*Tris-buffered saline (TBS)*

Compound	Amount
Tris HCl	1.58 g
NaCl	8.77 g
Sodium Azid	0.5 g
ddH <sub>2</sub> O	qs to 1.0 L

*TBS + 1% Tween*

Compound	Amount
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Tween 20	10 mL
TBS	990 mL

*TBS + 1.0% Tween*

Compound	Amount
Tween 20	1 mL
TBS	999 mL

*TBS + NP40*

Compound	Amount
NonIDET P40 (NP40)	30 mL
TBS	970 mL

Polyacrylamide gel electrophoresis:

1. Prepare resolving gel
  - a. Use 10% ethanol to clean glass plates
  - b. Set up plates in gel box
  - c. Prepare resolving gel by mixing reagents in the order as follows:

Compound	Amount
ddH <sub>2</sub> O	27.8 mL

Resolving gel buffer	20.1 mL
Acrylamide	31.8 mL
Persulfate	302 $\mu$ L
Temed	50 $\mu$ L

- d. Immediately pour gel into gel box, filling up to 1 cm below the comb line
- e. Tap gel box to remove any air bubbles: lift one edge of gel box about 1 cm and gently drop
- f. Immediately pipette 300  $\mu$ L ddH<sub>2</sub>O between each plate
- g. Allow gel to polymerize (approximately 1 hour)
- h. *Note: gel may be premade night before running gels. If this is done, pipette 500-700  $\mu$ L ddH<sub>2</sub>O between plates and set gel box in refrigerator (2-6 °C) overnight.*

2. Prepare stacking gel

- a. Use 10% ethanol to clean gel combs
- b. Place chromatography paper slips into space between gel plates to wick away all ddH<sub>2</sub>O (*without touching resolving gel*) and remove once all water is gone
- c. Prepare stacking gel by mixing reagents in the order as follows:

Compound	Amount
ddH <sub>2</sub> O	17.4 mL
Resolving gel buffer	7.6 mL
Acrylamide	5.0 mL
Persulfate	150.8 $\mu$ L

Temed	15.8 $\mu\text{L}$
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- d. Pour gel into gel box
- e. Immediately insert well combs between gel plates
- f. Allow gel to polymerize (approximately 1 hour)
3. Set up gel cartridges
  - a. Remove gels on at a time from gel box
  - b. Pull combs straight out of each gel
  - c. Rinse each gel with  $\text{dH}_2\text{O}$
  - d. Clamp two gels into a single gel cartridge
4. Prepare samples and standards
  - a. Mix as follows in microcentrifuge tubes:
    - i. Samples
      1. 2  $\mu\text{L}$  serum
      2. 18  $\mu\text{L}$   $\text{ddH}_2\text{O}$
      3. 20  $\mu\text{L}$  load buffer
    - ii. Recombinant human IGFBP-3
      1. 6  $\mu\text{L}$  standard
      2. 114  $\mu\text{L}$   $\text{ddH}_2\text{O}$
      3. 120  $\mu\text{L}$  load buffer
    - iii. Bovine
      1. 32  $\mu\text{L}$  serum
      2. 128  $\mu\text{L}$   $\text{ddH}_2\text{O}$

3. 160  $\mu$ L load buffer
  - b. Briefly centrifuge microcentrifuge tubes
  - c. Incubate tubes in 60 °C water bath for 10 minutes
  - d. Briefly centrifuge microcentrifuge tubes
5. Load gels
  - a. Fill gel cartridge with tank buffer
  - b. Load 20  $\mu$ L of sample or standard into each well
    - i. Do not use very first or last well and always load human standard into first lane used, followed by bovine standard
  - c. Load gel cartridges into running tank
    - i. Turn on water for cooling and turn on stir bar
6. Connect tank to voltage source and turn to 150 V. Run for 1.5 hours or until dye has reached bottom of gel.

#### Transfer to Nitrocellulose

1. Remove gels
  - a. Remove cartridges from tank
  - b. Separate gel plates
  - c. Cut stacking gel and excess gel away from resolving gel
2. Form transfer sandwich:
  - a. Chromatography paper
  - b. Gel
  - c. Nitrocellulose membrane (label gel ID in upper left corner with pen)

- d. Chromatography paper
  - e. *Make sure there are no bubbles between layers*
3. Place sandwiches in transfer cartridge (gel side down on black side of cartridge)
  4. Load transfer cartridges into transfer cell (black side of cartridge should face negative terminal)
    - a. Fill cell with Towbin buffer
    - b. Add cooling loop with running water
    - c. Add stir bar and place on stir plate – spin enough to circulate water but not create turbulence
  5. Connect to power supply and run at 45 V for 1.5 hours
  6. Remove nitrocellulose and dry between chromatography paper sheets (at least 20-30 min)  
*Proceed directly to next step or wrap gels individually in tin foil and freeze in -20 °C freezer until washing*

Tracer and Blocking:

*Before starting, make sure phosphor screen has been exposed to light for at least 5 hours*

1. Place each membrane in its own Tupperware container
2. Add 15 mL TBS + NP40
  - a. Place on shaker at 100 – 150 rpm for 15 min
  - b. Pour down drain
3. Add 15 mL TBS + 1% Tween
  - a. Place on shaker at 100 – 150 rpm for 2 hours
  - b. Pour down drain



4. Add 10 mL TBS + 0.1% Tween and enough  $I^{125}$ -IGF-I to obtain 300,000 cpm per blot
  - a. *Do not touch radioactivity directly to blot – tip Tupperware slightly and add to liquid away from blot*
  - b. Place on shaker at 100 – 150 rpm for 15 hours
  - c. Pour down drain with copious amounts of water
5. Add 15 mL TBS + 0.1% Tween
  - a. Place on shaker at 100 – 150 rpm for 15 min
  - b. Pour down drain
6. Repeat
7. Add 10 mL TBS
  - a. Place on shaker at 100 – 150 rpm for 10 min
  - b. Pour down drain
8. Repeat twice
9. Place blot on filter paper and dry (at least 20-30 minutes)
10. Wrap blots in saran wrap and place on phosphor screen
11. Incubate with screen in the dark for 18 hours

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